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# Kinin $B_1$ receptor is involved in mechanical nociception in a fibromyalgia-like model in mice

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## Abstract

Fibromyalgia-like models in mice induced by reserpine have opened a new avenue to understanding the molecular mechanisms behind this complex and incapacitating pain syndrome. The kinin B1 receptor (B1R) contributes to mechanical allodynia and acute coping behavior in mice with inflammatory and immunological disorders. This study has replicated previous data where amine depletion induced by reserpine significantly decreased the dopamine and serotonin levels in the prefrontal cortex (PFC), hippocampus (HPC), and spinal cord of mice. The animals subjected to the reserpine fibromyalgia model also showed decreased paw withdrawal threshold (PWT) and increased the immobility time in the forced swimming test (FST). Genetic ablation of B1R or pharmacological blockade by selective kinin B1R antagonist R-715 (acute i.p. treatment) counteracted the mechanical allodynia and increased immobility time induced by reserpine. However, neither pharmacological nor genetic inhibition of B1R reversed monoamine depletion. Our data confirm that reserpine induced a fibromyalgia-like phenotype in mice and reiterated the role of B1R on acute coping behavior and nociception modulation.

**Keywords:** Fibromyalgia, reserpine, kinin, B1 receptor, pregabalin

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## Introduction

Fibromyalgia is a complex and incapacitating pain syndrome characterized by widespread chronic musculoskeletal pain (1,2). Other clinical symptoms such as fatigue, depression, sleep disturbances, and cognitive dysfunction are frequently associated with this pathology (2–4), leading to impairment of patients' quality of life. The reduction of pain thresholds and pain sensitization is linked with decreased central monoamines levels (5,6). However, the mechanisms behind fibromyalgia pathophysiology are still unclear. Additionally, fibromyalgia treatment is complex, and the current pharmacological options available display limited effects and often have adverse side effects (7,8). Thus, further studies are necessary to clarify the mechanism involved in fibromyalgia's pathophysiology and discover a new pharmacological target to treat this pain syndrome.

Reserpine is an alkaloid that readily crosses the blood-brain barrier (BBB) and inhibits the storage of monoamines in vesicles, causing a depletion of neurotransmitters (e.g., dopamine and serotonin) in central and peripheral nerve terminals (demonstrating construct validity) (9,10). In preclinical studies, amine depletion by reserpine has been employed as a fibromyalgia-like model in mice and rats (9,11,12), in which the animals develop mechanical allodynia and increased immobility time in the forced swim test, FST (demonstrating face validity) (11,12). This alteration mimics some aspects of fibromyalgia symptoms in humans and facilitates the investigation of molecular mechanisms associated with disease and new pharmacological therapies. In this experimental paradigm, the results of repeated reserpine administration are attenuated by pregabalin, duloxetine, and pramipexole treatments (demonstrating predictive validity).

Our group has described the participation of Kinin B1R in both mechanical allodynia and acute coping behavior in mice subjected to immune-inflammatory challenge and in a mouse preclinical menopause model (13,14). Recently, Brusco *et al* have published an excellent study showing the participation of kinin receptors (B1R and B2R) in a fibromyalgia-like model induced by reserpine in adult male mice (12). This publication increases our confidence in the relevance of kinin B1R on molecular mechanisms involved in nociception and acute coping behavior in mice.

The present study was done independently, without any communication between the research groups. Here, we present our results that confirm the recent publication about the role of kinin B1R on mechanical allodynia and acute coping behavior in a preclinical model of fibromyalgia.

## Methods

In our study, we used adult (8 weeks of age at the beginning of study, weighing 25 to 30 g) C57BL/6 wild type (WT) and B1 receptor knockout male mice ($B1R^{-/-}$ (KOB1), UNIFESP-EPM). Animals were housed in groups of four or five per cage (30 x 20 x 13cm) under standard light conditions, temperature, and humidity (12 h light-dark cycle, 22 ± 1 °C, under 60 to 80 % humidity), with food and water provided *ad libitum*. The lights were on 7 am and off 7pm. Mice were kept in

microisolator cages equipped with inlet/outlet air filters and the cages were filled with autoclaved wood chip bedding. C57BL/6 mice were obtained from the Central animal facility from the Universidade Federal de Pelotas (UFPEL, Brazil) and acclimatized in our facility for two weeks before the experiments. KOB1 mice pups were born in our facility under standard mouse breeding colony management. The total number of animals used in this study were 96. Each experimental session included 4-6 animals of each treatment group, and the experiment was repeated 2-3 times. The investigators were blinded to treatment conditions during the behavior tests. All procedures were conducted following the Brazilian Council for Animal Experimentation (COBEA) guidelines, which comply with international laws and policies to investigate experimental pain in conscious animals. The protocols were approved by the local Ethical Committee (protocol number CEUA-PUCRS 12/00311), and all efforts were made to minimize animal suffering and reduce the number of animals used. The experiments were conducted between 8 am and 5 pm with randomization of the experimental groups throughout the day.

**Induction of fibromyalgia-like symptoms in mice**

The fibromyalgia-like model was accomplished as described previously (11,15). Briefly, amine depletion was induced by reserpine administration (0.25 mg/kg; Sigma-Aldrich, #50-55-5), given by subcutaneous route (s.c.), once a day for three consecutive days. A fresh solution of reserpine was prepared daily, dissolved in the vehicle (0.5% acetic acid solution in distilled water (v/v). Control groups received vehicle only, employing the same schedule of administration. The animals were subjected to behavioral tests on the 4$^{th}$ day. The dose and the time of reserpine treatment were selected based on previous studies (11,15,16).

**Mechanical allodynia**

The mechanical paw withdrawal threshold (PWT, also known as the von Frey test) measurement was carried out using the up-down paradigm, as described in the literature (11,17), with minor modifications. The mice were individually acclimated for one hour in elevated clear plexiglass boxes with a wire mesh floor to allow access to the right hind paw's plantar surface. After the first 30 min of acclimatization, the mice were removed from the box to undergo pharmacological treatment (i.p.) and immediately returned to the plexiglass box. The von Frey test begins after thirty minutes of injection, after which the mice have completed one hour of acclimatization in the plexiglass box. At the end of the von Frey test, the mice remained in the plexiglass box for 30 minutes and then were carefully transferred to an adjacent room to perform the FST.

Von Frey filaments of increasing stiffness (0.02–10g) were applied to the right hind paw plantar surface of the animals with pressure high enough to bend the filament. Tests were initiated with a 0.4g filament. The absence of a paw lifting after 5 s led to the use of the next filament with increased weight, whereas paw lifting indicated a positive response and led to the use of the next weaker filament. This paradigm continued for a total of 6 measurements, including the one before the first paw-lifting response had been made, or until four consecutives positive (assigned a score of 0.030) or four successive

negatives (assigned a score of 6.76) responses occurred. The mechanical paw withdrawal threshold response was then calculated as described previously (18), using the following formula: threshold 50% = log of the last hair used − (K. mean); where K is the constant based on the Dixon Table and refers to the mean difference (in log units) between stimuli (for mice 0.44). The paw withdrawal threshold was expressed in grams (g) and was evaluated before (baseline) and on the fourth day after reserpine injection. A significant decrease in paw withdrawal threshold compared to baseline values was considered mechanical allodynia.

**Forced Swim Test (FST)**

The mice were exposed to FST to assess acute coping behavior 30 min after the PWT. The experiments were carried out using cylinders (height 25 cm, diameter 18.5 cm) containing 17 cm height of clear water (23 ± 1 °C) for 6 min of forced swimming. Water in the cylinder was changed after each test to prevent carryover of alarm/stress substances. After testing, mice were dried off with a stack of paper towels and then placed into a cage with extra paper towels inside to facilitate the drying process. The mouse was judged to be immobile when it stopped struggling and floated in the water with minimal movements to keep its head above water. Immobility time was measured during the last 4 min period by a trained researcher blind to the treatment conditions (19,20).

**Pharmacological treatment**

To assess the involvement of B1R on the behavioral changes induced by reserpine, the animals were pre-treated with the selective B1R antagonist R-715 (0.5 mg/kg; i.p.; kindly provided by Dr. Fernand Gobeil - Department of Pharmacology, University of Sherbrooke, Qc, Canada). The control groups were treated with pregabalin (30 mg/kg; i.p.; Lyrica® - Pfizer) or saline (0.9%; i.p.). All treatments were administered 30 min before the Von Frey test. The doses and the treatment schedules were determined based on previous literature or pilot experiments (11,13,21)**.**

**Determination of neurotransmitters by *LC-MS/MS***

Variation in serotonin and dopamine levels in reserpine-induced fibromyalgia were analyzed in the prefrontal cortex, hippocampus, and spinal cord according to the method described by Gonzalez *et al.* (22), with minor modifications. The samples were dissected and immediately homogenized in a 15-fold volume of formic acid (0.1 M) and centrifuged at 20,000 x g for 20 min at 4 °C. Afterward, the supernatant was filtered (0.22 µm filter) and injected into the UHPLC 1290/MS 6460 TQQQ-Agilent (all HPLC components and the MassHunter software are from Agilent Technologies®). Chromatographic separations were performed using a Zorbax Eclipse Plus C18 2.1 x 50 mm 1.8-µm column. The flow rate of methanol (eluent A): formic acid 0.05% with 1mM of heptafluorobutyric acid (HFBA) (eluent B) mobile phase was 0.2 ml/min, with a column temperature of 30°C. A gradient was used, starting at 95 % of eluent B constant for 0.5 min, and subsequently decreasing to 0 % in 3.5 min. Five microliters of sample were injected into the UHPLC system. The monitored transitions were: dopamine (154>137 and 154>91) and serotonin (177>160). The results are expressed as the percentage of variation

(previously obtained in nanograms/gram of tissue) for dopamine and serotonin in relation to control vehicle/vehicle-treated groups (considered to be 100%).

**Statistical analysis**

The results are presented as the mean ± SEM. Differences in dopamine and serotonin levels were analyzed by a Student's t-test (between vehicle and reserpine groups, in each sample region). Two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc test was used to investigate the differences in the PWT (factors = time and treatment-group). In the case of interaction between factors, a one-way ANOVA followed by Bonferroni multiple comparison post hoc test was performed in the test session (supplemental table). In the FST, one-way ANOVA followed by Bonferroni multiple comparison post hoc test was performed. P-values less than 0.05 ($p < 0.05$) were considered significant. All tests were performed using Prism GraphPad Software version 8.0.2 (San Diego, USA). All data used in the present manuscript is available under CC-BY license in FigShare (DOI:10.6084/m9.figshare.13087442).

**Results and Discussion**

**Reserpine decreases the levels of dopamine and serotonin in the prefrontal cortex, hippocampus, and spinal cord.**

To confirm the construct and face validity of reserpine inducing a fibromyalgia-like model in mice (11,12), we assayed dopamine and serotonin levels after three consecutive reserpine injections. Our results showed that on the fourth day after reserpine treatment (24h after last injection), there was a significant reduction of serotonin (Figure 1B) in the PFC (WT, $t = 5.597$, $df = 5$; $p = 0.0019$ and KOB1, $t = 4.283$, $df = 6$; $p = 0.0052$, N = 3-4), HPC (WT, $t = 8.537$, $df = 5$; $p = 0.0004$ and KOB1, $t = 13.27$, $df = 6$; $p = 0.0001$, N = 3-4) and spinal cord (WT, $t = 20.37$, $df = 5$; $p = 0.0001$ and KOB1, $t = 13.60$, $df = 6$; $p = 0.0001$, N = 3-4). Similar data were observed in dopamine levels (Figure 1C) in the PFC (KOB1, $t = 4.991$, $df = 5$; $p = 0.0001$, N = 3-4), HPC (WT, $t = 6.207$, $df = 5$; $p = 0.0016$ and KOB1, $t = 3.607$, $df = 6$; $p = 0.0113$, N = 3-4) and spinal cord (WT, $t = 10.74$, $df = 5$; $p = 0.0001$ and KOB1, $t = 12.11$, $df = 6$; $p = 0.0001$, N = 3-4), but not in PFC-WT group ($t = 1.122$, $df = 5$; $p = 0.3128$, N = 3-4). Both strains showed a similar profile in the decrease of serotonin and dopamine after reserpine injection. A similar decrease in serotonin level was observed in both strains (WT *versus* KOB1, one-way ANOVA = $F (5, 18) = 6.235$) in PFC ($t = 1.351$, $p = 0.5802$), HPC ($t = 0.878$, $p = 0.99$) and spinal cord ($t = 2.107$, $p = 0.1481$, Figure 1B). On the other hand, a significant difference in dopamine levels were observed (WT *versus* KOB1, one-way ANOVA = $F (5, 18) = 17.92$) in PFC ($t = 5.250$, $p = 0.0002$), HPC ($t = 2.640$, = 0.499) and spinal cord ($t = 4.288$, $p = 0.0013$, Figure 1C). The data agree with the previous publication, in which the depletion of monoamines causes a sensitization to mechanical stimulus and nociceptive behavior in rodents (11,12,16). In the clinic, pain and mood disorders are common side effects of reserpine therapy (23). Thus, a decrease of monoamines in central and peripheral neurons is an important alteration in fibromyalgia (11,23). However, it is still unclear what

the mechanism of the decrease of monoamines is. Further studies are necessary to determine if the decrease of monoamines is a cause or consequence of fibromyalgia.

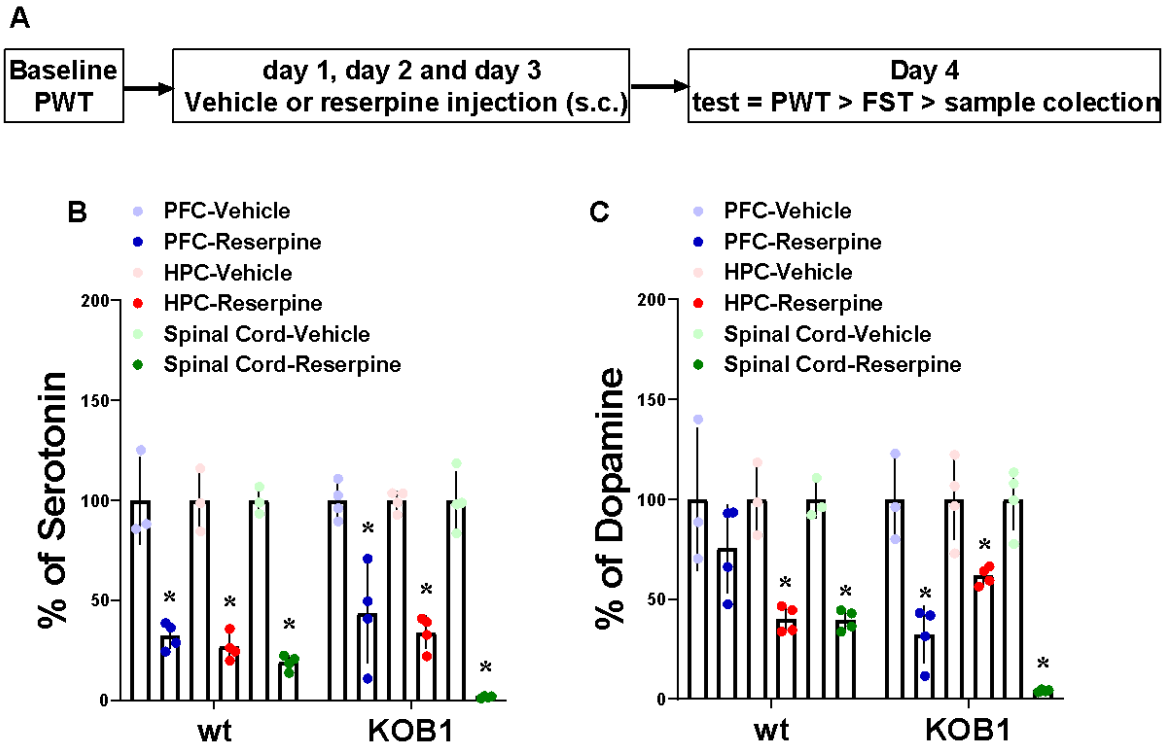


**Figure 1. Effect of repetitive reserpine injection on serotonin and dopamine levels.** (A) Timeline of the experimental approach. Percentage of serotonin (B) and dopamine (C) levels on the prefrontal cortex (PFC), hippocampus (HPC), and spinal cord one day after the last administration of reserpine. Each column represents the mean ± SEM of 3-4 animals per group. *P < 0.05 and significantly different from the vehicle for each sample region (Student's *t* test). wt = wild type.

**Pharmacological and genetic ablation of the kinin B1R reduces reserpine-induced mechanical allodynia and increased of immobility time in FST.**

In the present study we also demonstrate that acute treatment with pregabalin (30 mg/kg; i.p.; 30 min. before behavioral test) or with B1R antagonist R-715 (0.5 mg/kg, i.p; 30 min. before behavioral test) significantly inhibited the mechanical allodynia induced by reserpine (interaction during test = F (3, 36) = 25.40; pregabalin: t = 7.459, p = 0.0001 and R-715: t = 9.959, p = 0.0001; N = 8-12; Figure 2A). However, only the treatment with R-715 significantly reduced immobility time in the FST (F (3, 36) = 37.42; R-715: t = 6.594, p = 0.0001; N = 8-12; Figure 2C). Similar to pharmacological inhibition of the kinin B1R, the B1R receptor knockout mouse (KOB1R) showed a lack of fibromyalgia-related mechanical hypersensitivity (interaction during test = F (3, 37) = 8.49; KOB1-reserpine: t = 7.142, p = 0.0001; N = 10-11; Figure 2B) and increased the immobility time in the FST (F (3, 37) = 10.10, KOB1-reserpine: t = 3.577, p = 0.0059, N = 10-11; Figure 2D).

Here, we reproduce data from previous studies and confirm that inhibition of B1R counteracts mechanical allodynia and acute coping behavior in fibromyalgia-like models induced by reserpine (11,12,16). However, we identified some differences in the reserpine-induced fibromyalgia-like model in mice. In the current study, we used a reserpine dose of 0.25 mg/kg (s.c. injection, for three consecutive days), which was effective in inducing nociception and acute coping behavior. This is different from the study of Brusco and collaborators, who decided to use a high dose (1 mg/kg) of reserpine in the same schedule of treatment. In our previous work (11), reserpine (0.25 mg/kg) impaired locomotor activity, assayed in an open field arena, which is corroborated by other studies (24–26). Such locomotor impairment induced by reserpine was not observed by Brusco and collaborators (12), and was also not reported in other publications (27). The divergent results concerning locomotor activity modulated by reserpine may be related to the animal's strain and age. Further investigation is necessary to better understand the impact of motor alteration induced by reserpine on behavioral changes associated with fibromyalgia-like models in rodents.

The FST is the most common behavioral paradigm used to characterize “depressive-like behavior” in rodents. However, this very questionable paradigm only measures acute coping behavior (28,29). Considering the fact that motor activity impairment can directly change the performance and time of swimming in FST (30), we need to be cautious in saying that reserpine induces acute coping (despair behavior) in mice. However, the increase of immobility time in FST induced by reserpine was attenuated by fluoxetine treatment (31), confirming the model's predictive validity to assess compounds with antidepressant-like effects. Also, reserpine significantly decreased sucrose preference in rats (32), modeling anhedonic behavior observed in patients with major depression. Altogether, reserpine can induce molecular and behavioral changes in rodents that mimic some pathophysiological aspects of fibromyalgia, making possible the development of new pharmacological strategies.

In conclusion, our study added more evidence to the role of kinin B1R on mechanical allodynia and acute coping behavior induced by reserpine.

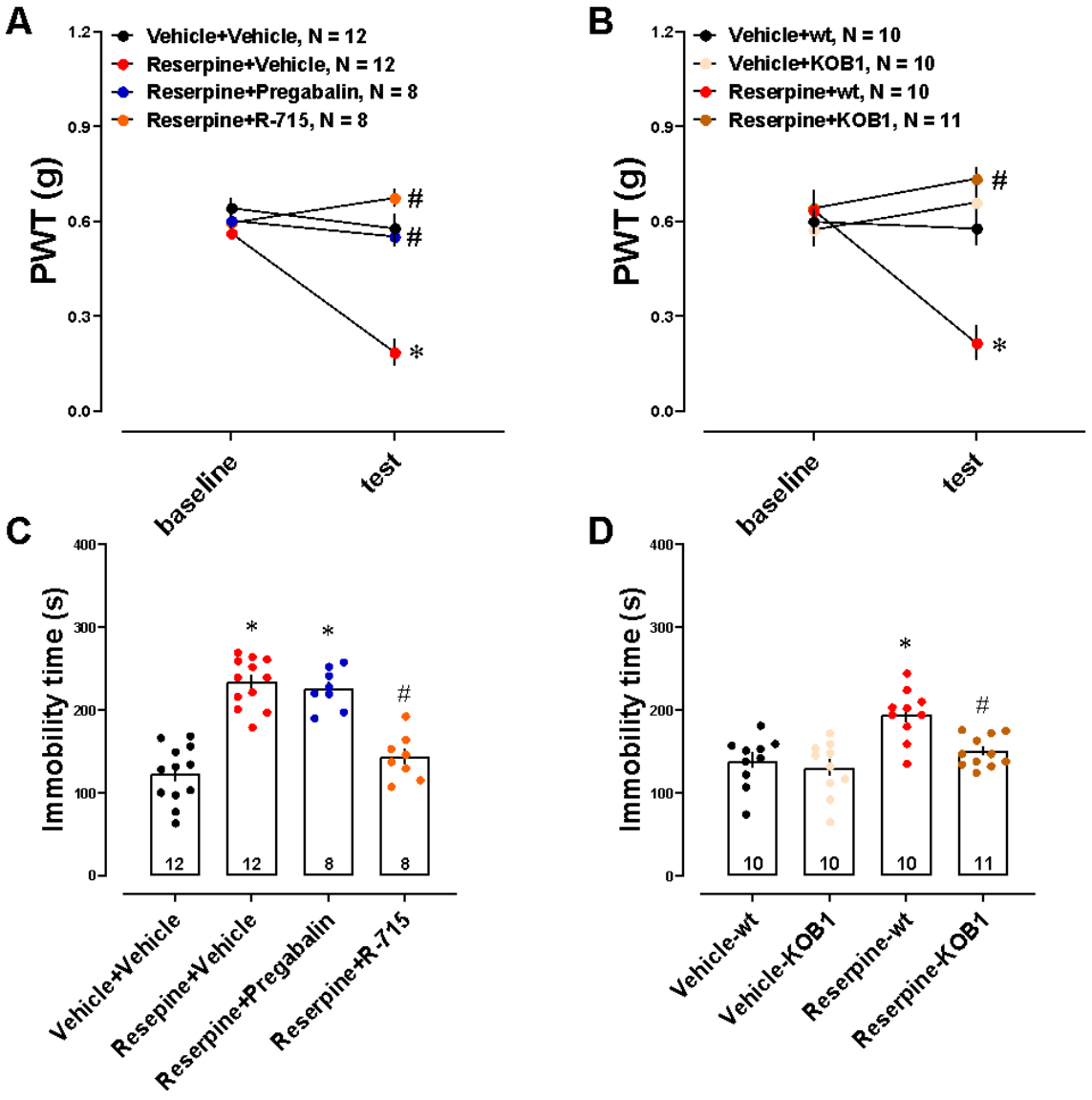


**Figure 2. $B_1$ receptor showed a protective effect against mechanical allodynia and depressive-like behavior induced by reserpine.** (A and B) The mechanical allodynia threshold was analyzed using the von Frey test and (C and D) immobility time in the forced swimming test. Effects of treatment with pregabalin (30 mg/kg) or R-715 (0.5 mg/kg), both by administered i.p., injected 30 min before behavioral tests, are shown. Each column represents the mean ± SEM of 8-12 animals per group. *P < 0.05 and significantly different from the vehicle+vehicle or vehicle-wt group and #P < 0.05 and significantly different from the reserpine+vehicle or reserpine-wt group Statistical analysis was performed by 2-way ANOVA, followed by Bonferroni post hoc test, factors = time and treatment-group (A and B) or one-way ANOVA, followed by Bonferroni post hoc test (C and D). N = number of animals per group.

## Authors' contribution

ISM designed the study, performed the experiments, collected, analyzed, and interpreted data, and wrote the manuscript draft. VMA and PO performed the experiments, and collected and analyzed data. APAD and MMC designed the study, analyzed and interpreted data, and reviewed the manuscript draft.

**Conflict of interest and funding**

None of the authors declares any conflict of interest. This work was supported by grants from Brazilian research agencies (CAPES), National Council for Scientific and Technological Development (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and FINEP Research Grant "Implantação, Modernização e Qualificação de Estrutura de Pesquisa da PUCRS" (PUCRS INFRA) # 01.11.0014-00.

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